of *N*-(*R*,*S*-2-hydroxypropyl)-L-valine. (Current mechanistic studies of the *N*-alkyl Edman method indicate that racemization of the amino acid occurs.)

An important point is the choice of GC column and, further, for the analysis of hydroxyalkylvaline-PFPTHs, that the use of the column for other purposes should be avoided. Silylation of OH groups of PFPTs has been used.[21,22]

Derivatized samples have been shown to give reproducible results in analysis after years of storage at −20°, protected from light, at least with respect to measurement of HOEtVal–PFPTH at levels ≥100 pmol/g.

Calibration

It is essential to establish calibration curves from samples with a composition as similar as possible to those of the globin samples to be measured.[12] In the case of HOEtVal–PFPTH calibration, the samples to be derivatized have consisted of 50 mg of low-background globin (from a nonsmoker) with addition of different amounts of the calibration-standard globin and of the internal-standard globin dissolved in formamide. However, when measurement of background levels is intended, other proteins available in pure form may be used together with standard globins.[12]

It has been found that in solutions of too low globin concentrations the alkylated standard globins are subjected to losses, which could show up as nonlinear calibration curves. This may be counteracted by adding carrier globin with low background levels to the standard globin solutions to a concentration of ~5 mg globin/ml formamide.[12]

Because calibration curves are linear and samples can be reanalyzed with reproducible results for long periods of time, a check of the reproducibility is obtained by including one calibration sample in every new sample series to be derivatized and analyzed.

If free ($^2H_4$)HOEtVal is used as an internal standard it has to be considered that, because of the higher pK_a, it reacts more slowly than HOEtVal in globin.[21] For the same reason derivatization of free amino acids is done in more alkaline medium (1-propanol : 0.5 *M* $KHCO_3$, 1 : 2) (pH 8.6). It seems that ($^2H_4$)EO-alkylated globin may be used as an internal standard for other hydroxyalkylvalines and that, e.g., ($^2H_3$)-methylated globin could be used as an internal standard for other alkylvalines.[23] However, for exact quantitation proper calibration is required to allow for differences

[22] E. Bailey, P. B. Farmer, Y.-S. Tang, H. Vangikar, A. Gray, D. Slee, R. M. J. Ings, D. B. Campbell, J. G. McVie, and R. Dubbelman, *Chem. Res. Toxicol.* **4**, 462 (1991).

[23] M. Törnqvist, S. Osterman-Golkar, A. Kautiainen, M. Näslund, C. J. Calleman, and L. Ehrenberg, *Mutat. Res.* **204**, 521 (1988).

in response factors, fragmentation patterns in MS analysis, and possibly in yield.

Sensitivity and Accuracy

The contributions to the uncertainty under the instrumental conditions described have been determined in a few series of background level HOEtVal measurements.[11] The coefficients of variation (CV) for derivatization, injection, and reading were found to be 4.7, 7.8, and 4.5%, respectively, corresponding to a total CV of 10% in measurements close to the detection level, ~10 pmol/g. In a ring test of HOEtVal-PFPTH determinations different laboratories achieved results that, considering the low level (10–100 pmol/g), were in acceptable agreement (interlaboratory CV, 28%).[21]

By GC-MS-MS analysis of samples prepared as described above the sensitivity was increased by a factor of 10, thus reaching a detection level below 1 pmol/g Hb, which corresponds to an injected amount of about 1 fmol (Törnqvist *et al.*, in preparation). Since in this case the background noise is eliminated a further increase of the sensitivity is possible by using larger samples.

Acknowledgment

The author thanks Anna-Lena Magnusson for excellent technical assistance. The Swedish Environmental Protection Agency has been the main sponsor of the work described.

[44] Tobacco-Specific Nitrosamine–Hemoglobin Adducts

By STEPHEN S. HECHT, STEVEN G. CARMELLA, and SHARON E. MURPHY

Tobacco-specific nitrosamines are a group of carcinogens formed by nitrosation of nicotine and other tobacco alkaloids.[1,2] Two of these compounds, *N'*-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), have been implicated in the etiology of cancers of the oral cavity, lung, pancreas, or esophagus caused by use of tobacco products.[3,4] Based on the amounts of NNN and NNK in tobacco

[1] D. Hoffmann and S. S. Hecht, *Cancer Res.* **45**, 935 (1985).
[2] S. S. Hecht and D. Hoffmann, *Carcinogenesis (London)* **9**, 875 (1988).
[3] S. S. Hecht and D. Hoffmann, *Cancer Surv.* **8**, 273 (1989).
[4] D. Hoffmann and S. S. Hecht, *in* "Handbook of Experimental Pharmacology" (C. S. Cooper and P. L. Grover, eds.), p. 63. Springer-Verlag, Heidelberg, 1990.

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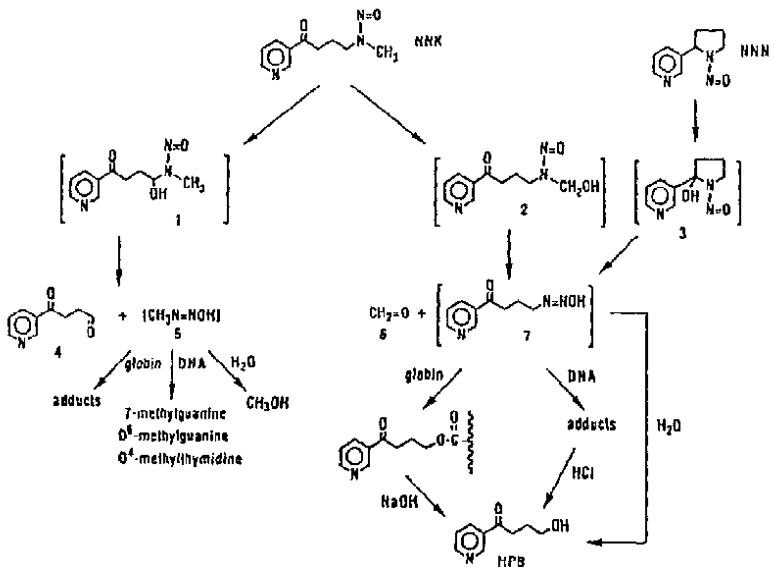

FIG. 1. Metabolic pathways leading to the formation of DNA and globin adducts of NNK and NNN.

products, human exposure to these carcinogens is similar to the doses that cause tumors in laboratory animals. The method described here can potentially provide information not only on an individual's exposure to NNN and NNK, but also on the metabolic activation of these carcinogens to intermediates that bind to cellular macromolecules.

NNN and NNK are converted to highly electrophilic intermediates by enzymatic hydroxylation of the carbons α- to the *N*-nitroso group. In the case of NNK, α-methylene hydroxylation yields methane diazohydroxide (**5**, Fig. 1), which methylates DNA and hemoglobin. α-Methyl hydroxylation produces 4-(3-pyridyl)-4-oxobutane diazohydroxide (**7**, Fig. 1), which pyridyloxobutylates DNA and hemoglobin. Hydroxylation of NNN at its 2′-position, adjacent to the pyridine ring and *N*-nitroso groups, yields the same pyridyloxobutylating agent that is produced by methyl hydroxylation of NNK. These reactions are illustrated in Fig. 1 (**1–7**). Extensive studies on the metabolism and DNA binding of NNK have implicated both DNA methylation and pyridyloxobutylation as important factors in carcinogenesis by this

nitrosamine.[1,2,5–11] DNA pyridyloxobutylation is also likely to play an important role in NNN carcinogenesis.[1,2,12,13]

Pyridyloxobutylation of hemoglobin was first observed in rats treated with NNK and NNN.[14] Alkylation occurs on the protein, not the protoporphyrin. Treatment of hemoglobin or globin with NaOH at room temperature releases 20–40% of the bound material as 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB).[14–16] The adduct that releases HPB on this mild base hydrolysis is an ester, formed by reaction of either aspartate, glutamate, or the terminal carboxyl group of globin with the diazohydroxide.[17] Other studies have shown that HPB does not react with globin to produce an adduct that releases HPB on mild base hydrolysis.[16] Thus, the release of HPB from globin is a measure of the extent of metabolic activation of NNK or NNN to 4-(3-pyridyl)-4-oxobutane diazohydroxide.

Dose–response studies carried out with NNK in rats have demonstrated that the formation of the ester adduct that releases HPB is linear over a dose range of four orders of magnitude.[15] Comparative studies of globin and DNA pyridyloxobutylation in rats treated with NNK have shown that both parameters increase with dose. However, DNA pyridyloxobutylation in liver and lung does not vary in a linear fashion with dose, and the relationship between DNA and globin pyridyloxobutylation by NNK is thus complex.[15]

The methodology described here was developed to quantify levels of HPB released on mild base hydrolysis of human hemoglobin.[18] It is known

[5] S. S. Hecht, K. G. Jordan, C.-I. Choi, and N. Trushin, *Carcinogenesis (London)* **11**, 1017 (1990).
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[9] L. A. Peterson, R. Mathew, S. E. Murphy, N. Trushin, and S. S. Hecht, *Carcinogenesis (London)* **12**, 2069 (1991).
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[15] S. E. Murphy, A. Palomino, S. S. Hecht, and D. Hoffmann, *Cancer Res.* **50**, 5446 (1990).
[16] L. A. Peterson, S. G. Carmella, and S. S. Hecht, *Carcinogenesis (London)* **11**, 1329 (1990).
[17] S. G. Carmella, S. S. Kagan, and S. S. Hecht, *Chem. Res. Toxicol.* **5**, 76 (1992).
[18] S. G. Carmella, S. S. Kagan, M. Kagan, P. G. Foiles, G. Palladino, A. M. Quart, E. Quart, and S. S. Hecht, *Cancer Res.* **50**, 5438 (1990).

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that individuals vary widely in their abilities to enzymatically activate or detoxify carcinogens such as NNN and NNK.[19] Presumably, those individuals who activate these carcinogens extensively would have a higher risk for cancer development than those who were more proficient in detoxification. Quantitation of hemoglobin adducts of NNN and NNK could thus provide an index of their uptake and metabolic activation.

The method consists of four major steps: (1) isolation of hemoglobin and treatment with base to release HPB; (2) solvent partitions to enrich HPB; (3) derivatization of HPB to its pentafluorobenzoate, which can be detected with great sensitivity and selectivity by capillary column gas chromatography–mass spectrometry with negative-ion chemical ionization detection and selected ion monitoring (GC-MS-NICI-SIM); and (4) quantitation by GC-MS-NICI-SIM.

Materials and Methods

Chemicals

HPB, [4,4-D_2]HPB (internal standard), HPB-pentafluorobenzoate, [4,4-D_2]HPB-pentafluorobenzoate, and HPB-tetrafluorobenzoate (external standard) are synthesized,[18] or can be obtained from the authors on request. Pentafluorobenzoyl chloride is obtained from Aldrich Chemical Co. (Milwaukee, WI). Pentanophenone and hexanophenone are purchased from Pierce Chemical Co. (Rockford, IL). Trimethylamine (TMA) hydrochloride and dichlorodimethylsilane are obtained from Sigma Chemical Co. (St. Louis, MO).

Instrumentation

Solvent concentration is performed with a Model SVC200H SpeedVac centrifugal concentrator (Savant Instruments, Farmingdale, NY). With this model, heat (43°) may be applied to the sample. All centrifugations are performed using the SpeedVac (v = 1750 rpm). HPLC is carried out with a system consisting of two Model 510 pumps, a Model 680 automated gradient controller, a Model 440 fixed-wavelength UV/visible detector, all from Waters Division of Millipore (Milford, MA), and a Model 7125 injector from Rheodyne (Cotati, CA). GC-MS-NICI-SIM is performed with a Hewlett-Packard Model 5988A instrument (Hewlett-Packard Co., Palo Alto, CA).

[19] A. Castonguay, G. D. Stoner, H. A. J. Schut, and S. S. Hecht, *Proc. Natl. Acad. Sci. U.S.A.* 80, 6694 (1983).

Procedures to Minimize Contamination and Low Recovery

The levels of HPB released from hemoglobin are typically less than one part per billion, and the actual amounts quantified may range from 1 to 100 pg/sample. Therefore, special precautions are necessary to avoid contamination and loss of analyte. Analyses are carried out in a dedicated area or laboratory, with dedicated glassware and HPLC equipment. Organic solvents are HPLC or GC-MS grade and are used without further purification.[20] Sodium hydroxide solution (1 *N*) is freshly prepared from NaOH pellets and HPLC-grade H_2O. Reagent-grade concentrated HCl is diluted with purified H_2O to 1 *N* and stored in glass bottles.

Only silanized glassware is used; 24 50-ml disposable borosilicate centrifuge tubes (Kimble, Toledo, OH) are silanized as follows. The tubes are filled with 5% (v/v) dichlorodimethylsilane in CH_2Cl_2 and incubated for 30 min. The silanizing reagent is decanted and the tubes are filled with methanol and allowed to stand for 30 min. The tubes are then rinsed twice with methanol, covered with foil, and allowed to dry overnight.

Silanize 4-ml borosilicate screw cap vials (Baxter, McGaw Park, IL) and 250-μl microvials ("limited-volume inserts," National Scientific Co., Lawrenceville, GA) as follows. The vials are placed in a vacuum desiccator along with a 20-ml scintillation vial containing 2 ml of dichlorodimethylsilane. The desiccator is flushed with N_2 for 3 min. A vacuum is applied for 1 min, and then the desiccator is flushed again with N_2 for 1 min to distribute the silanizing reagent. The vacuum is reapplied and the sealed desiccator is allowed to stand for at least 1 hr. The desiccator is then flushed with N_2. The vials are removed, rinsed twice with methanol, once with CH_2Cl_2, then covered with foil and dried at 180° for 2–3 hr.

All glassware is rinsed with the solvent to be used before each procedure.

Collection of Blood and Isolation of Hemoglobin

Blood is collected in 10-ml heparinized Vacutainers (Baxter, McGaw Park, IL). It is recommended to use at least 5–10 ml human blood for each assay. The blood is centrifuged at 900 *g* for 10 min at 4°. The supernatant is discarded and the packed red blood cells (RBCs) are washed three times

[20] It is critical that solvents be of highest purity. In our experience one batch of HPLC-grade CH_2Cl_2 contained 1% methanol. This resulted in 0% recovery of [4,4-D_2]HPB, because the methanol reacted with all the derivatizing agent.

PM30011926 13

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by centrifugation with 0.9% saline (w/v). The RBC are lysed by mixing with 3 volumes of ice-cold H_2O on a vortex mixer for 5 min. After 15 min at 0°, KH_2PO_4 buffer, 0.67 *M*, pH 6.5, is added to make the final buffer concentration 0.2 *M*. The resulting mixture is centrifuged at 25,000 *g* for 30 min at 4°. The supernatant is removed and dialyzed 20 to 24 hr against 10–20 volumes of distilled H_2O, using Spectra/Por 4 cellulose dialysis tubing, with a molecular weight cutoff of 12,000–14,000 (Baxter, McGaw Park, IL). The dialysis H_2O is changed four times.

Treatment of Hemoglobin Solutions with Base

Hemoglobin solution (5–10 ml) is poured into a 50-ml glass centrifuge tube, and 1 *N* NaOH is added to a final concentration of 0.15 *N*. The mixture is stirred on a vortex mixer for 5 min and then placed in a sonicator (Branson 1200, Baxter) for 1 hr at room temperature. After sonication, the globin is precipitated by neutralizing the solution with 1 *N* HCl. Then 50 pg (299 fmol) [4,4-D_2]HPB is added from an aqueous solution (5 pg/μl). This solution is prepared by the addition of 5 μl of a stock solution of [4,4-D_2]HPB (1.2 ng/μl in isopropanol) to 1.2 ml H_2O. The globin and [4,4-D_2]HPB are mixed by inverting the capped tube several times. The globin is pelleted by centrifugation for 10 min. The supernatant, containing HPB, is transferred to a fresh 50-ml glass centrifuge tube.

Solvent Partitioning to Enrich HPB

The supernatant is adjusted to pH 2 within HCl. Under these conditions, HPB is protonated.[21] The supernatant is extracted with an equal volume of CH_2Cl_2 by shaking for 5 min in an automatic shaker. The CH_2Cl_2 layer is separated by centrifugation and discarded. This procedure is repeated. Then, the aqueous layer containing protonated HPB is extracted twice with equal volumes of hexane, using the same procedure. The hexane extracts are discarded. The pH of the aqueous phase is adjusted to 7, which deprotonates the pyridine ring of HPB. It is extracted three times with equal volumes of CH_2Cl_2 as described above. It is important in this step not to carry over any H_2O. The pooled CH_2Cl_2 layers are then

[21] We are investigating the use of PrepSep C_{18} extraction columns (Fisher Scientific) in place of partitioning. The supernatant containing HPB is applied to the column, washed with 5 ml H_2O, then HPB eluted with 1 ml methanol. The methanol is added to 9 ml H_2O and HPB is extracted with an equal volume of CH_2Cl_2 once. The CH_2Cl_2 is concentrated to dryness on a SpeedVac.

concentrated to dryness in the SpeedVac concentrator. The concentration step requires approximately 90 min, with heat.

Derivatization of HPB to HPB-Pentafluorobenzoate

A solution of TMA in hexane is prepared as follows. TMA hydrochloride (240 mg) is placed in a 50-ml glass centrifuge tube and to this is added 2.4 ml of 1.0 *N* NaOH and 17.6 ml of H_2O, followed by 20 ml of hexane. The mixture is shaken for 1 min and allowed to stand for 5 min. The hexane layer is transferred to a 20-ml scintillation vial and dried by addition of 2–3 g of Na_2SO_4. It is shaken and allowed to stand for 5 min, and the hexane solution of TMA is decanted.

For derivatization, reagents are added sequentially with vortexing to the 50-ml centrifuge tube containing the fraction enriched with HPB, as follows: 0.5 ml of CH_2Cl_2, 1 ml of TMA in hexane, and 0.5 ml of 14 m*M* pentafluorobenzoyl chloride in CH_2Cl_2. The tube is agitated on a mechanical shaker for 30 min at room temperature. The solution is then transferred to a silanized 4-ml vial. The tube is washed with 0.5 ml of CH_2Cl_2 and the washings are added to the solution in the 4-ml vial. The CH_2Cl_2 is removed by evaporation in the SpeedVac, without heat, for 30 min.

The derivatized sample is then purified by HPLC. A 10 cm × 4.6 mm Partisil 5 ODS 3 cartridge column (Whatman Co., Clifton, NJ) is used. The column is washed successively with H_2O, methanol, and 65 : 35 (H_2O : methanol) (v/v) for 1 hr each at 1 ml/min, and then overnight in 65 : 35 (H_2O : methanol) at 0.2 ml/min.

The retention time of HPB-pentafluorobenzoate is marked with pentanophenone and hexanophenone. This marker mixture is prepared by mixing 1 ml each of tetrahydrofuran (THF) and methanol with 5 μl each of pentanophenone (10 mg/ml methanol) and hexanophenone (10 mg/ml methanol). Of this mixture, 70 μl is injected on the column with a solvent program as follows: 65 : 35 (H_2O : methanol), 10 min; then linear to 30 : 70 in 15 min; hold at 30 : 70 for 9 min; then 0 : 100 for 9 min; then return to 65 : 35 in preparation for the next sample. The flow rate is 1 ml/min. Under these conditions, the elution times of the pentanophenone and hexanophenone markers are 29 and 31 min, respectively, and the elution time of HPB-pentafluorobenzoate is 30 min.

The derivatized sample is mixed with 70 μl of the marker mix, vortexed, and allowed to stand for 10 min prior to injection. Standing for 10 min will allow time for methanol to scavenge excess pentafluorobenzoyl chloride. The resulting methyl ester elutes at 25 min. The region eluting be-

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tween the apices of the pentanophenone and hexanophenone peaks is collected in 4-ml vials. The samples are concentrated to dryness in the SpeedVac, with heat, for 2.5 hr. The dried samples are stored at −80° until analysis by GC-MS-NICI-SIM. Each sample is transferred to a microvial by rinsing the 4-ml vial three times with 6 drops of THF, stirring 2 min each time on a vortex mixer.

Analysis by GC-MS-NICI-SIM

Solutions of HPB-pentafluorobenzoate, [4,4-D_2]HPB-pentafluorobenzoate (internal standard), and HPB-tetrafluorobenzoate (external standard) are prepared in ether, immediately prior to analysis. They are used to generate a standard curve. The concentration of the external standard is 30 fmol/μl. The concentrations of HPB-pentafluorobenzoate and [4,4-D_2]HPB-pentafluorobenzoate range from 5 to 30 fmol/μl. The inclusion of an external standard provides a measure of the sensitivity of detection by GC-MS for a particular sample. We have found that the sensitivity varies between standards and samples, and among samples.

A 0.25-mm i.d. × 30-m 50% methylphenylsilicone (DB-17) bonded phase column (0.15-μm film thickness), connected to a 0.32-mm i.d. × 2-m fused silica retention gap is used. The column is connected to the retention gap with a silica press-fit union (Restek Corp., Bellefonte, PA). The seal is reinforced with polyimide resin. Injections are carried out with a septumless on-column injector (J&W Scientific, Folsom, CA). Injections are made with a 10-μl syringe equipped with a 5-inch fused silica needle. The needle is placed into the retention gap such that 2 inches extend into the GC oven. The sample is deposited at an injection speed of 1 μl/sec onto the retention gap with the oven equilibrated at 35°. The injection port temperature is 35° and, after injection, the oven is programmed as follows: 35° for 1.0 min; then 20°/min to 150°; then 4°/min to 215°; then hold for 8 min. The flow rate is 1 ml/min helium measured at 35°.

Ether (10 μl) containing 300 fmol of the external standard, HPB-tetrafluorobenzoate, is added to the conical microvial containing the derivatized sample. Then, the walls of the vial are washed with approximately 3 μl of ether. The volume of the ether solution is then measured by taking it up in a 10-μl syringe. The solution is placed back into the vial, and 50% of it is taken up with a fused silica needle syringe containing enough ether to make the final injection volume 6 μl. It is injected on the column and the temperature program is initiated. The mass spectrometer is operated in the NICI mode with a methane pressure of 0.8 Torr, ionizing energy of 120 eV, and a source temperature of 150°. The molecular ions of HPB-pentafluorobenzoate (*m/z* 359), [4,4-D_2]HPB-pentafluorobenzoate (*m/z* 361), and HPB-tetrafluorobenzoate (*m/z* 341) are monitored.

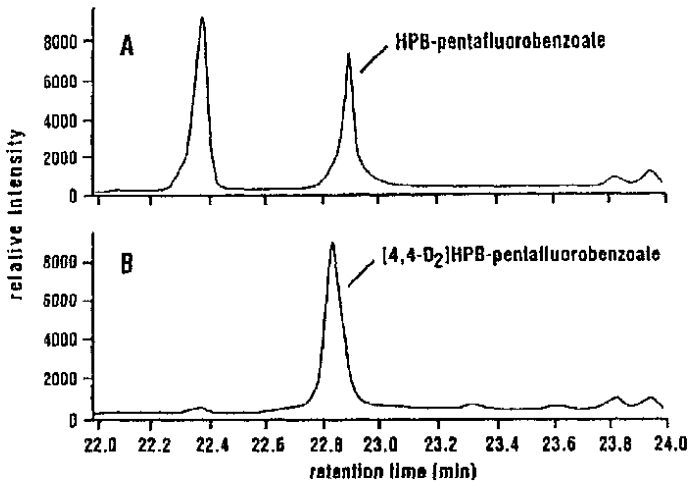

FIG. 2. GC-MS-NICI-SIM chromatograms obtained using the method described here, applied to hemoglobin from a smoker; (A) SIM at *m/z* 359 (molecular ion of HPB-pentafluorobenzoate) and (B) SIM at *m/z* 361 (molecular ion of internal standard, [4,4-D_2]HPB-pentafluorobenzoate).

Calculations

The amount of HPB in each sample is determined by multiplying the amount of [4,4-D_2]HPB added by the ratio of the area of the peaks at 359 and 361 mass units. A correction is made for any difference in GC-MS detector response between HPB and [4,4-D_2]HPB. The level of HPB is expressed as femtomoles/gram of Hb. Hemoglobin is analyzed as cyanomethemoglobin (Sigma kit No. 525A). Percent recovery may be calculated using the external standard in conjunction with these data.

Results and Comments

This method for the analysis of HPB is both sensitive and specific. The sensitivity results from the electron capture properties of HPB-pentafluorobenzoate, which make it readily detectable by NICI detection. The detection limit for standard HPB-pentafluorobenzoate, monitored by SIM at its molecular ion of *m/z* 359, is approximately 100 amol. The

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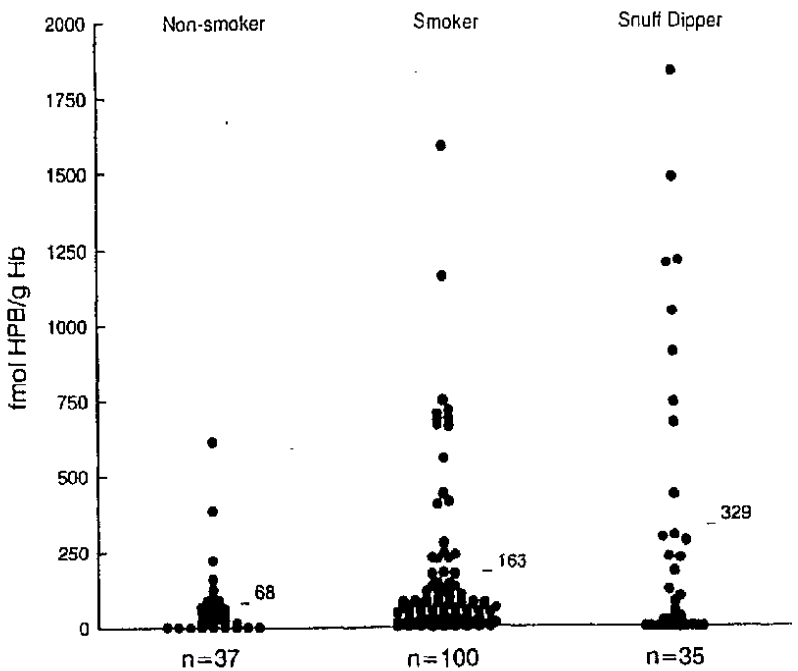

FIG. 3. Data obtained on analysis of hemoglobin of nonsmokers, smokers, and snuff dippers for HPB released on base hydrolysis. Data are from ongoing studies conducted in our laboratory by P. G. Foiles and S. Akerkar, as well as from published studies.[18]

specificity results from the outstanding chromatographic resolution achieved with capillary column GC and the SIM of the particular molecular ion of the analyte. The internal standard, [4,4-D_2]HPB, not only allows quantitation of HPB in the sample, but also provides a reliable GC retention time marker for HPB. We have found that the peak for the molecular ion of [4,4-D_2]HPB, monitored by SIM at *m/z* 361, generally elutes 0.04 min (typically at 22.85 min) before the peak for the molecular ion of HPB, monitored by SIM at *m/z* 359 (typically eluting at 22.89 min). This is illustrated in Fig. 2.

The high sensitivity of the method results in detection of some HPB-pentafluorobenzoate in H_2O blanks. The source of this apparent contamination is unknown, but it typically amounts to approximately 60 fmol per 10 ml H_2O (1 ppt). Samples are analyzed in sets of 8–10. Each set includes an H_2O blank as well as a positive control sample, which is a dilution of hemoglobin from a rat treated with [5-^{3}H]NNK. The level of HPB was

determined by analysis of [5-^{3}H]HPB to be 500 fmol/g Hb. These negative and positive controls serve as quality control monitors for the analysis. The amount of HPB in the negative control is subtracted from the amount in a given sample. We do not consider a sample to be positive unless the amount of HPB in that sample is at least twice background.

The precision of the method has been described.[18] Replicate values of a given sample run in the same set of samples on the same day have a relative standard deviation of less than 5%. Replicate values of a given sample run in different sets of samples on different days have a relative standard deviation of approximately 25%. The higher relative standard deviation obtained in multiple sets probably results in part from differences in GC-MS responses.

Some representative values for HPB released from human hemoglobin are summarized in Fig. 3. The levels of HPB releasing adducts detected in nonsmokers are generally lower than those of hemoglobin adducts of any other carcinogen examined to date, which is probably a reflection of the tobacco-specific nature of NNK and NNN.[18] Most of the HPB detected in the blood of nonsmokers is likely associated with background from unknown sources, but some may be due to exposure to NNK and NNN in environmental tobacco smoke. In smokers, we have consistently observed elevated levels of HPB-releasing adducts in about 20% of the samples analyzed. The reasons for this intriguing observation are presently under investigation. Preliminary data suggest that levels of HPB-releasing adducts in smokers are not a reflection of the amount of NNK and NNN in a cigarette, but rather may be influenced mainly by metabolic activation and detoxification parameters. Levels of the HPB-releasing adducts in snuff dippers generally appear to be higher than those in smokers. This finding requires further study.

Acknowledgments

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